BENZOPORPHYRIN DERIVATIVE AND LIGHT-EMITTING DIODE FOR USE IN PHOTODYNAMIC THERAPY: APPLICATIONS OF SPACE LIGHT-EMITTING DIODE TECHNOLOGY

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Abstract

Photodynamic therapy (PDT) is a cancer treatment modality that recently has been applied as adjuvant therapy for brain tumors. PDT consists of intravenously injecting a photosensitizer, which preferentially accumulates in tumor cells, into a patient and then activating the photosensitizer with a light source. This results in free radical generation followed by cell death. The development of more effective light sources for PDT of brain tumors has been facilitated by applications of space light-emitting diode array technology; thus permitting deeper tumor penetration of light and use of better photosensitizers. Currently, the most commonly used photosensitizer for brain tumor PDT is Photofrin®. Photofrin® is a heterogeneous mixture of compounds derived from hematoporphyrin. Photofrin® is activated with a 630 nm laser light and does destroy tumor cells in animal models and humans. However, treatment failure does occur using this method. Most investigators attribute this failure to the limited penetration of brain tissue by a 630 nm laser light and to the fact that Photofrin® has only a minor absorption peak at 630 nm, meaning that only a small fraction of the chemical is activated. Benzoporphyrin Derivative Monoacid Ring A (BPD) is a new, second generation photosensitizer that can potentially improve PDT for brain tumors. BPD has a major absorption peak at 690 nm, which gives it two distinct advantages over Photofrin®. First, longer wavelengths of light penetrate brain tissue more easily so that larger tumors could be treated, and second, the major absorption peak means that a larger fraction of the drug is activated upon exposure to light. In the first part of this project we have studied the tumoricidal effects of BPD *in vitro* using 2A9 canine glioma and U373 human glioblastoma cell cultures. Using light emitting diodes (LED) with a peak emission of 688 nm as a light source, cell kill of up to 86 percent was measured in these cell lines by tumor DNA synthesis reduction. The effectiveness of BPD against tumor cells *in vitro* thus established, we have taken the first step toward determining its effectiveness *in vivo*. The second part of this project consisted of experiments performed to determine the maximum tolerated dose (MTD) of both BPD and LED light. At a light dose of 100 J/cm^2, skin damage and neurotoxicity were seen at a BPD dose of 1.0 mg/kg, but not at a dose of 0.75 mg/kg. When BPD remained constant at 0.75 mg/kg, skin damage was seen at light dosages of 125 J/cm^2, 150 J/cm^2 and 175 J/cm^2. One dog also died at a light dose of 175 J/cm^2. Further studies will be needed to determine the effectiveness of BPD against tumor cells *in vivo*.

INTRODUCTION

Photodynamic therapy (PDT) is a relatively new adjuvant treatment for brain tumors in research and in clinical practice (Ji 1992, Kaye 1987, Kostron 1987, Laws 1981, Lindsay 1991, Muller 1990, Powers 1991, Schmidt 1994, and Whelan 1993). The cytotoxic photodynamic effect is based upon the interaction of a localized photosensitizer, light and oxygen (Henderson 1992). Photofrin® is currently the most commonly used photosensitizer for PDT of brain tumors. Photofrin® is a heterogeneous mixture of hematoporphyrin which accumulates preferentially in brain tumors (Hill 1990, Kaye 1988, Whelan 1993, and Whelan 1994). The absorption spectrum of photofrin® is relatively broad with two significant absorption peaks: a major absorption peak at 390 nm and a minor absorption peak at 630 nm (Kaye 1988). Traditionally, red laser light with a wavelength of 630 nm has been used to activate photofrin®, the decreased absorbance of this wavelength by photofrin® being compensated for by the increased penetrance of brain tissue demonstrated by longer wavelengths of light. Red laser light is frequently produced by using an Argon ion or KTP/YAG laser beam that is converted by a dye module to 630 nm. This conversion is inherently costly and inefficient, but allows for light to be delivered by fiberoptics. For non-fiberoptic delivery of light, other light sources could be a potentially useful alternative.

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Light-emitting diodes (LED) are one such light source that may prove to be an effective alternative to lasers for PDT. LED's have been frequently used to emit low power, broad spectrum light of 25-30 nm bandwidth for photosynthesis research in plants (Barta 1992, Bula 1991, and Tennessen 1994). LED lamps traditionally consist of an array of semiconducting LED chips. In recent years, improvements in semiconductor technology have substantially increased the light output of LED chips. A novel type of LED chip is based on the semiconductor Aluminum Gallium Arsenide (AlGaAs). These LED chips can be manufactured to emit light with a peak wavelength between 630 nm and 940 nm (Bula 1991, and Tennessen 1994). This range of wavelengths overlaps with the absorption spectrum of photosensitizers used for PDT of brain tumors.

The purposes of this study were to test the cytotoxicity of the LED/BPD combination against 2A9-canine glioma and U373 human glioblastoma cells *in vitro*, and to determine the maximum tolerated dose (MTD) of both LED-derived light and BPD in dogs.

METHODS

Photodynamic effect *in vitro* was tested using an LED array to direct light into culture dishes containing either BPD-sensitized cells, or control cells. DNA synthesis was then measured to assess cell damage. *In vivo* photodynamic therapy studies were performed using an LED probe inside a fluid filled balloon. The balloon was placed into the posterior fossa of adult dogs, some of which had received BPD and some of which were non-photosensitized controls. Varying doses of BPD and LED light were administered to determine the maximum tolerated doses of both.

Photodynamic Studies of Glioma *In Vitro*

Assays of photodynamic effect were conducted using a flat LED array to direct light into culture dishes containing brain tumor cells in both the presence and absence of BPD; the ability of the cells to perform DNA synthesis was then measured. Red light for PDT *in vitro* was produced by a Q-Beam photosynthesis lamp (Quantum Devices, Inc., Barneveld, WI, USA). The emitting surface of this lamp measured 6 x 10 cm and contained 198 LED chips on top of a ceramic sink and a cooling fan. The intensity of the emitted red light was modulated via a ten turn potentiometer which controlled the current to the LED chips. The glioma cells for the *in vitro* experiments were exposed to 100 J/cm^2. The temperature at the emitting surface did not exceed 37° C at any time during the experiments.

Canine glioma clones were grown in a monolayer tissue culture in P100 tissue culture dishes at 37° C in a humidified, five to ten percent CO_2 atmosphere, as previously described (Schmidt, 1996). Cultures were harvested by gently dislodging the cells from their substratum with a Pasteur pipette for *in vitro* assay. Canine glioma cells were then plated in the 1.6 cm well of a 24 plate. Following twenty four hours of attachment, BPD was added in concentrations of 5, 10, 20 and 60 ng/ml in five percent dextrose solution. This concentration did not produce any dark toxicity in our cell line. All experiments were done in sixlettes. Light-only control cells received an equal volume of five percent dextrose solution without BPD. After 30 minutes of incubation in the dark, the cells were exposed to light from an LED lamp as described above.

Approximately two hours after completion of light exposure, treatment cell viability was assessed by measuring uptake of ^{3}H-Thymidine in DNA. Cells were exposed to 1 μCi of ^{3}H-Thymidine. After four hours of ^{3}H-Thymidine exposure, the medium was removed. The monolayer of glioma cells was washed with cold phosphate buffered saline solution. Then the acid soluble material was extracted with a ten percent trichloroacetic acid (TCA) solution for fifteen minutes. The TCA solution was subsequently removed and the monolayer transferred into a scintillation vial and thoroughly mixed with scintillation fluid. Radioactivity was counted in a Beckman LS 6000TA liquid scintillation spectrometer.

Photodynamic Therapy Studies *In Vivo*

In order to deliver LED light *in vivo* and compare it to the conventional laser balloon adapter, an LED probe was constructed (Figure 1). The LED probe consists of a 10 cm hollow steel tube that has at its tip 144 LED chips

arranged in a cylinder. The core of the tube contains three channels. One channel contains insulated wires which provide electricity for the LED tip. A second channel contains sterile water which acts as a cooling fluid when circulated around the tip. The third channel provides access for the 0.1 percent intralipid fluid used to inflate the balloon at the tip of the probe. The pump for the cooling fluid and the power supply are in a portable base unit. Power output of the LED tip is adjusted via a potentiometer on the base unit which controls current flow. The temperature of the tip was continuously monitored and was not allowed to exceed 35° C at any time during the experiment. The balloon of the LED probe was inflated with a 0.1 percent intralipid solution. Balloon diameter ranged from 2 cm to 5 cm. The tip of the LED probe was placed in the center of the balloon. In this configuration, the balloon is known to scatter light uniformly, with light irradiation varying by no more than twenty percent of the average when measured at any point on the balloon's surface (Schmidt, et al, 1996). The total power output was 1.0 watt at the tip and was kept constant for all measurements.

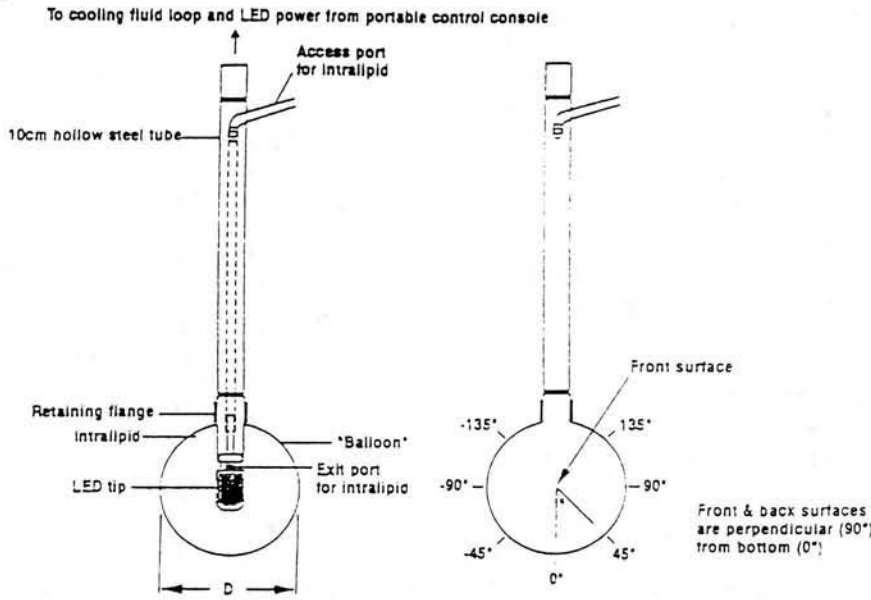

FIGURE 1. The LED balloon applicator in a schematic overview.

Adult mongrel dogs each weighing approximately 20 kg were used for *in vivo* PDT. All animals were intubated and placed under general anesthesia using halothane/nitrous oxide. The head of the canine was securely fixed to a head frame and maximally flexed. Using a posterior approach, the suboccipital bone and C1 vertebra were exposed. A wide craniectomy was performed using a power drill. A Y-shaped dural incision exposed the cerebellum, lower brainstem and upper cervical spinal cord. The balloon applicator with a diameter of 2 cm was placed into the posterior fossa on the brainstem. Self-retaining retractors were used to secure the balloon applicator to prevent excessive pressure from being place onto the brainstem.

To determine the MTD of BPD, seven dogs were used. These dogs all received 100 J/cm^2 of LED light, with the dosage of BPD being the control variable. One dog received no BPD, three dogs received 0.75 mg/kg of BPD and three dogs received 1.0 mg/kg of BPD. The MTD was defined as the dose given to the group of canines that preceded the group with a 50 percent clinical toxicity rate.

To determine the MTD of LED light, eleven dogs were used. These dogs all received the MTD of BPD determined above, and varying amounts of LED light. BPD was administered three hours prior to light application; there was no BPD only group. Three dogs received 125 J/cm^2 of LED light, three received 150 J/cm^2, four received 175 J/cm^2 and one received 200 J/cm^2. These dogs were then split into two groups. Those receiving less than or equal to 150 J/cm^2 of light and those receiving more than 150 J/cm^2 of light.

Canines from both portions of the *in vivo* study were monitored for skin toxicity and neurotoxicity, and were given an MRI to assess damage.

RESULTS

The LED light/BPD combination showed photodynamic effects *in vitro* which resulted in reduced DNA synthesis in experimental cells versus non-photosensitized control cells. *In vivo* results indicate that there is a definite maximum tolerated dose of both LED light and BPD over which skin and neurotoxicity results.

Photodynamic Studies of Glioma *In Vitro*

There was no significant difference between glioma cells that received LED light only and cells that had no treatment. LED light exposure of BPD sensitized cells resulted in a significant reduction in the amount of DNA synthesis performed by 2A9 canine glioma and U373 human glioma cells. The effects of LED light and BPD are illustrated in Figure 2 and Table 1.

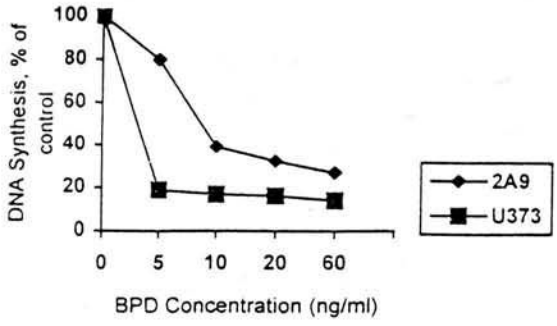

FIGURE 2. Photodynamic Effect of BPD In Vitro.

TABLE 1. DNA Synthesis (Percent of Control) at Varying BPD Concentrations.

BPD Conc. (ng/ml)	DNA Synthesis 2A9 Canine Glioma	(% of control) U373 Human Glioma
0	100 ± 3.77	100 ± 9.45
5	79.59 ± 4.26	19.05 ± 1.28
10	39.02 ± 1.44	16.91 ± 1.91
20	32.28 ± 1.52	16.18 ± 1.11
60	26.97 ± 1.13	14.1 ± 1.49

Photodynamic Therapy Studies *In Vivo*

When 100 J/cm^2 of LED light was used in the absence of BPD, no skin toxicity or neurotoxicity resulted. When 100 J/cm^2 of LED light and .75 mg/kg of BPD were used together, again, no toxicity resulted. In contrast, when 100 J/cm^2 and 1.0 mg/kg of BPD were used, two dogs displayed skin toxicity, and three dogs displayed transient neurotoxicity. None of the dogs that received 100 J/cm^2 of LED light in combination with any amount of BPD died or showed signs of damage on MRI. Table 2 summarizes these results.

TABLE 2. Results From Dogs Receiving 100 J/cm^2 of LED Light.

Light Dose	BPD Dose	Number of Dogs	Cases of Skin Toxicity	Cases of Neuro-toxicity	Deaths	MRI-visible damage
100 J/cm^2	0.0 mg/kg	1	0	0	0	none
100 J/cm^2	.75 mg/kg	3	0	0	0	none
100 J/cm^2	1.0 mg/kg	3	2	3	0	none

When 0.75 mg/kg of BPD was used in combination with doses of LED light less than or equal to 150 J/cm^2, toxicity of any kind was uncommon. In these nine dogs, we saw only two cases of skin toxicity, and no neurotoxicity, death or MRI-visible damage. In the five dogs receiving 0.75 mg/kg of BPD and more than 150 J/cm^2 of light, we saw two cases of more severe skin toxicity and one death. Table 2 summarizes these results.

TABLE 3. Results From Dogs Receiving 0.75 mg/kg of BPD.

Light Dose	BPD Dose	Number of Dogs	Cases of Skin Toxicity	Cases of Neuro-toxicity	Deaths	MRI-visible damage
$\leq$150 J/cm^2	.75 mg/kg	9	2	0	0	none
>150 J/cm^2	.75 mg/kg	5	2	0	1	none

DISCUSSION

The development of new light sources and light delivery devices is of critical importance for improving photodynamic therapy. In this study an LED probe with an inflatable tip was used instead of the traditional laser light source. The concept of using a fluid filled balloon to photo-illuminate a tumor cavity after maximal resection was originally put forward by Muller and Wilson (Muller 1985, Muller 1986, Muller 1987, Muller 1990, and Wilson 1986). They developed a laser balloon adapter in which the red laser light, produced at the power module, is delivered via a fiberoptic to the center of a fluid filled balloon. The fluid inside the balloon serves as a light scattering medium. In addition, the inflated balloon prevents the collapse of the resection cavity which could potentially prevent light from reaching sensitized tumor cells. The LED probe is similar to the laser probe in that a fluid filled balloon is used to scatter light. However, the LED probe does not require a fiberoptic for light delivery, as the light is produced at the tip of the probe in the center of the balloon. This arrangement eliminates the approximately 50 percent loss of power seen between the laser light source and the target tissue when using the laser probe, making the LED probe the more efficient method.

Another advantage of using LED probes over lasers for PDT is the increased range of wavelengths that can be produced by them. As mentioned previously, red laser light is frequently produced using an Argon ion or KTP/YAG laser beam, and then converted to the 630 nm wavelength needed to activate photofrin® by a costly process: LED arrays can be manufactured to emit a wide range of wavelengths, eliminating the need for this process. One can simply order the LED needed for activation of the chosen photosensitizer. The availability of longer wavelength light sources allows for the use of newer, second generation photosensitizers such as BPD. As previously mentioned, BPD has several characteristics which make it more attractive for PDT of brain tumors than photofrin®. BPD has a major absorption peak at both 415 and 690 nm, which gives it two distinct advantages. First, longer wavelengths of light penetrate brain tissue more easily. This means that the 690 nm wavelength light could activate photosensitizer in the center of tumors that could not be reached by a 630 nm light. Second, the absorption peak at 690 nm is a major one which means that a larger fraction of the BPD is activated upon exposure to light than that which would be activated by exposing photofrin® to light. BPD also causes the formation of nearly five times the amount of singlet oxygen molecules as the equivalent amount of photofrin®, can be administered much closer to the time of light exposure and causes shorter periods of skin toxicity in patients receiving it (Aveline 1994). These effects are summarized in Table 4.

TABLE 4. Characteristics of Photofrin and BPD

	Photofrin®	BPD
absorption peak	365 nm (major) and 630 nm (minor)	415 nm (major) and 690 nm (major)
quantum yield singlet oxygen	0.16	0.78
light exposure	18 to 72 hours	15 min to 4 hours
skin toxicity	4 weeks	24 hours

CONCLUSIONS

In vitro photodynamic therapy using BPD and LED light is effective against canine and human glioma cells. Administering a concentration of 10 ng/ml of BPD 30 minutes prior to light application reduces DNA synthesis by more than 50 percent. *In vivo*, the MTD of BPD is 0.75 mg/kg and the MTD of LED light is 100 J/cm^2.

Photodynamic Studies of Glioma *In Vitro*

Based on our results, we conclude that photodynamic therapy, using the LED light source and BPD photosensitizer combination, is effective against 2A9 canine glioma and U373 human glioma cells *in vitro*. We also conclude that a BPD concentration of 10 ng/ml or higher will result in a greater than 50 percent reduction in DNA synthesis, and that 30 minutes is adequate time to allow for uptake of the BPD *in vitro*. In addition, human glioma cells were more sensitive than canine glioma cells *in vitro*.

Photodynamic Therapy Studies *In Vivo*

The results of this portion of the study indicate that the MTD of BPD is 0.75 mg/kg and the MTD of LED light is between 100 and 150 J/cm^2 in dogs. Minor local skin damage is encountered at these doses.

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